

## Seed transmission of *Verticillium dahliae* in olive as detected by a highly sensitive nested PCR-based assay

MUWAFFAQ R. KARAJEH

Department of Plant Protection and IPM, Faculty of Agriculture, Mu'tah University, Karak 7, Jordan

**Summary.** To determine whether the spread of *Verticillium dahliae* to new olive growing areas can be seed-borne, fruit samples of *V. dahliae*-infected symptomatic and asymptomatic trees of two olive cultivars (Shimlali and Nabali) were randomly collected in November and December 2003 from two olive-growing areas in Jordan. Seeds were excised from the fruits and some of the seeds were sown to produce progeny seedlings. Both seeds and the seedlings were tested for *V. dahliae* infection using standard plating and a nested polymerase chain reaction (PCR)-based assay that used primers from the internal transcribed spacer (ITS) regions of nuclear ribosomal RNA (rRNA) genes. The sensitivity of the nested PCR-based assay was investigated by amplifying the crude DNA of conidia. The incidence of *V. dahliae* infection in seeds and seedlings was significantly higher with the nested PCR-based assay than with the plating procedure in both symptomatic and asymptomatic trees of both olive cultivars. Infection rates were significantly higher in symptomatic than in asymptomatic trees and, in general, higher for the cv. Shimlali than the cv. Nabali. The incidence of *V. dahliae* infection in the seedlings was significantly higher than that in the seeds. The expected DNA fragments were amplified from all the concentrations of *V. dahliae* conidial suspensions used ( $2 \times 10^4$ ;  $2 \times 10^3$ ;  $2 \times 10^2$ ; 20 and 2 conidia  $\mu\text{l}^{-1}$ ) indicating that the assay was highly sensitive. Olive seeds of the two cultivars transmitted *V. dahliae* to the progeny seedlings in different percentages up to a maximum of 35%. Infected olive seed contributes significantly to pathogen dissemination.

**Key words:** culture plating, DNA fingerprints, disease diagnosis, vascular wilt.

### Introduction

Olive (*Olea europea* L.) is the most widely grown fruit tree in Jordan, occupying more than 80% of the total area planted with fruit trees (Statistical Yearbook, 2003). Verticillium wilt, caused by *Verticillium dahliae* Kleb., is one of the most serious diseases affecting olive worldwide. It causes severe losses to olive plantations in the whole Mediterranean region (Heale, 2000). In Jordan, wilt on olive was first reported in the 1980s, and in the last 20

years it has spread throughout the main olive-growing areas of Jordan (Mamluk *et al.*, 1984; Naser, 1996; Masoud and Karajeh, 2003). Several factors may account for the spread of the disease, including the use of infected planting material, the establishment of new orchards in infected soils or close to affected crops, and the establishment of irrigated orchards with an excessive tree density (Naser, 1996). The dissemination of *V. dahliae* by seed has been documented in many crop species (Toit *et al.*, 2005; Vallad *et al.*, 2005).

The pathogen colonizes the xylem tissues and may even invade the inflorescence, and subsequently the developing fruit and seed. Systemic invasion occurs when successive generations of conidia are produced, then transported through the

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To correspond with the author:  
Fax: +962 3 2323 154  
E-mail: muwaffaq@mutah.edu.jo

xylem transportation stream to the aerial parts of infected plants (Goth and Webb, 1981). Seed transmission of *V. dahliae* could therefore be responsible for its spread throughout planting area, in addition to other mechanisms of spread resulting from human activity (Vallad *et al.*, 2005).

The rapid and consistent detection of *V. dahliae* in olive plants is of great importance in the management of Verticillium wilt. Early detection would help to prevent the spread of the pathogen to new areas (Carder *et al.*, 1995).

Several methods for detecting latent *Verticillium dahliae* infection in olive are currently used, the most common is pathogen isolation by plating infected plants on culture media. However, this method is time consuming, and has a low detection potential especially with latent infections, resulting in a high proportion of false negatives, and it is difficult with a slow growing fungus such as *V. dahliae* (Dhingra and Sinclair, 1985; Plasencia and Bantari, 1997). Genetic fingerprinting of the causal agent by polymerase chain reaction (PCR)-based techniques could be the only practical method for disease diagnosis before symptom development and disease spread (Martin *et al.*, 2000). When properly optimized, PCR is highly sensitive even with very small amounts of tissue (Nazar *et al.*, 1991). In previous studies, a molecular procedure was developed for the early diagnosis of latent *V. dahliae* infection (Masoud and Karajeh, 2003). This procedure included the extraction of PCR-quality DNA from *V. dahliae* and the use of *V. dahliae*-specific primers from internal transcribed spacer (ITS) regions of nuclear ribosomal RNA (rRNA) genes in a nested-PCR protocol (Masoud, 2002; Masoud and Karajeh, 2003).

During a visit to an olive nursery at Al-Halabat location in Jordan, it was found that some 15% of olive seedlings cv. Shimlali (a seedling rootstock cultivar) showed vascular wilt. *V. dahliae* was isolated from these seedlings but it was not known whether the infection had come from the seeds (i.e. the mother plant) or from the bed soil. To our knowledge, there is no report on *V. dahliae* transmission through the seed. The main objectives of this study were to determine whether olive seed was a carrier of *V. dahliae*, using a nested PCR-based assay, and to assess the transmission of *V. dahliae* to progeny seedlings, which are usually grown from seed rather than from stem cuttings.

## Materials and methods

### Fungal cultures

Twelve isolates of *V. dahliae* (MU1, MU6, MU12, MU17, MU19, MU23, MU28, MU30, MU39, MU41, MU47, and MU70), that had been collected from olive trees in Jordan and were maintained in pure cultures on potato dextrose agar medium (PDA) at 22°C in darkness (Masoud and Karajeh, 2003), were used in this study. The pathogenicity of the isolates was confirmed by testing on three plant species, tomato, eggplant, and cucumber (Masoud and Karajeh, 2003), as well as on olive (Karajeh and Al-Raddad, 1997).

### Determination of the seed-borne nature of *V. dahliae* in olive

Three hundred mature fruits were collected and individually packed in plastic bags (25 fruits/tree) from six *V. dahliae*-infected symptomatic and six infected asymptomatic trees of the cultivar Nabali from the Mu'tah olive-growing area, Karak, and of the cultivar Shimlali, from an olive nursery in the Al-Halabat area, Azraq in Jordan during November and December 2004. Immediately after collection, fruits were individually surface disinfected in a sodium hypochlorite solution (1% v:v) for 4 min, followed by three rinses in sterile distilled water. The seeds were then excised from the other parts of the fruit (epicarp, mesocarp and endocarp). The seeds and the other parts of each fruit were individually cross-sectioned into four segments and then placed separately in Petri dishes on a medium semi-selective for *V. dahliae* (Ausher *et al.*, 1975). Samples were incubated at 22°C in darkness until the mycelium began grow out of the seed pieces.

To assess the possibility of *V. dahliae* being transmitted to progeny seedlings through the seed a further 300 mature fruits were collected, sampled and treated as above. Excised seeds of this batch (with its endocarp) were sown in a 2:1 (v:v) mixture of autoclaved clay and sand (1 h at 121°C) into small, 350 ml seedling pots. The progeny seedlings were grown in a growth room (25±3°C with a 16-h day) for 8 weeks. Pathogen isolation was done after 8 weeks from planting; the stems of the developed seedlings were individually cross-sectioned at the crown area and cut into 1 cm segments, placed in separate Petri dishes on the semi-selective medium, and incubated at 22°C in darkness until mycelium began to grow out of the seedling pieces.

#### DNA extraction from *V. dahliae* isolates and from infected plant tissues

The CTAB procedure of DNA extraction (Rogers and Bendich, 1985) used in the present study was that which had first been followed by Masoud and Karajeh (2003) with minor modifications to isolate DNA from *V. dahliae* isolates and from infected olive stem sections. Agar plugs (0.5 cm diameter) of *V. dahliae* isolates were cut from the active growing edges of cultures, placed in 100 ml flasks containing 50 ml Czapek's dox broth and incubated under continuous orbital shaking (200 rpm min<sup>-1</sup>). After 14 days the fungal biomass was harvested by vacuum filtration using Whatman No.1 filter paper and washed with sterile distilled water. The fungal biomass (mycelia and spores) (about 0.2 g fresh weight) was ground to a fine powder in liquid nitrogen using a porcelain mortar and pestle. The powder was homogenized in 1.5 ml preheated (65°C) extraction buffer (50 mM Tris-HCl [pH 8]; 700 mM NaCl; 10 mM EDTA-Na<sub>2</sub>; 2% CTAB; 0.2% [v:v] 2-mercaptoethanol, and 0.4% [w:v] polyvinylpyrrolidone PVPP). The homogenate was divided into two 1.5 ml microtubes and incubated at 65°C for 15 min with occasional mixing, followed by extraction with 0.6 ml chloroform-isoamyl alcohol (24:1). After 5 min centrifugation at 14,000 g, the top aqueous phase was transferred to a new tube containing 2 µl RNase A (10 mg ml<sup>-1</sup> water) and incubated for 15 min at room temperature. Isopropanol (0.3 ml) was added to each microtube and the mixture was incubated overnight at -20°C. The DNA pellet obtained after 5 min centrifugation at 14,000 g was washed with 1 ml of 70% ethanol, air dried, and dissolved in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The quality and the concentration of the DNA were estimated using agarose gel electrophoresis and with a spectrophotometer at a wavelength of 260 nm (Sambrook *et al.*, 1989).

For plant-DNA extraction, seed samples (10 seeds/sample) and xylem portions (0.2 g) taken from 10 randomly selected samples from the crown area of the progeny seedlings were individually ground in liquid nitrogen using a Phillips screwdriver. They were processed with the same protocol as that used for DNA extraction from the fungal cultures, but the extraction buff-

er had 0.5% (v:v) 2-mercaptoethanol and 1% (w:v) PVPP, and an additional step of protein precipitation, using 0.5 volume of 3M sodium acetate pH 5.2, was added after the RNase A treatment.

#### Nested PCR Amplification

The nested PCR primers were used to amplify the DNA extracted from the fungal cultures, the excised seeds, and the progeny seedlings. Nested PCR amplification consisted of two successive reactions. The first amplification round was accomplished with the primer pair (NESF18S, 5'-CCTCATAACCCCTTTGTGAACC-3' and NESR28S, 5'-CCGAGGTCAACCGTTGCCG-3') from the highly conserved DNA sequences of the 18S and 28S genes (Fig. 2) that flank the ITS region (White *et al.*, 1990; Nazar *et al.*, 1991; Volossiuk *et al.*, 1995), producing a DNA product about 480 bp in size, while the second amplification round was carried out with the *V. dahliae*-specific ITS primers (FVD, 5'-GGTCCATCAGTCTCTCTG-3' and RVD, 5'-TCCGATGCGAGCTGTAAC-3') designed by Masoud (2002), producing a DNA product about 330 bp in size. One microliter of the product from the first amplification round was transferred to a fresh tube containing the mixture for the second round. The conditions and parameters in the second round were the same as in the first round. The PCR was performed in a total volume of 25 µl, with each reaction containing the following: 0.2 mM dNTPs (an equal molar mixture of dATP, dGTP, dCTP and dTTP), 0.5 unit of *Taq* DNA polymerase, 0.25 mM of each forward and reverse primer, IX PCR buffer (10×: 500 mM KCl, 15 mM MgCl<sub>2</sub>, 100 mM Tris-HCl [pH 9 at 25°C] and 1 Triton X-100), 1 µl of the final DNA extract (around 50 ng). The materials for the PCR including the dNTPs, 10× PCR buffer and *Taq* DNA polymerase were purchased from Promega (Madison, WI, USA). A negative PCR control (water) was used in each round to detect any contamination with template DNA and olive DNA extract so as to insure there was no amplification of plant DNA (Sambrook *et al.*, 1989). The PCR cycles were programmed in an Eppendorf Mastercycler (Hamburg, Germany) with one initial step of DNA denaturation at 94°C (2 min) followed by 35 cycles, each consisting of denaturation at 95°C

(30 s), annealing at 56°C (30 s), and extension at 72°C (30 s). The final step was extension for 3 min at 72°C.

The sensitivity of the nested PCR-based procedure in detecting *V. dahliae* was evaluated as follows: a conidial suspension was prepared by washing a 5-day-old culture (isolate MU39) of *V. dahliae* in a few drops of nuclease-free water, and the wash was collected in a 1.5 ml microtube. The number of conidia in the suspension was adjusted to  $2 \times 10^4$  conidia  $\mu\text{l}^{-1}$  using a hemacytometer slide that indicated a high counting reproducibility between laboratories (Rodríguez-Tudela *et al.*, 2003). The conidial suspension was vigorously homogenized for 15 s with a vortex mixer before making several serial ten-fold dilutions ( $2 \times 10^4$ ,  $2 \times 10^3$ ,  $2 \times 10^2$ , 20 and 2 conidia  $\mu\text{l}^{-1}$ ), which were carefully examined under a light microscope. One microliter of each conidial dilution was used directly for DNA amplification without DNA extraction using the same protocol of nested PCR.

#### DNA electrophoresis and documentation

The PCR products were analyzed by DNA electrophoresis in agarose gel (Sambrook *et al.*, 1989) with  $0.5 \times$  TBE buffer ( $10 \times$  buffer of 0.9 M Tris, 0.9 M boric acid, and 20 mM EDTA). Each gel contained at least one lane of 100 bp DNA ladder (Promega) as a molecular size marker. DNA was stained with ethidium bromide and visualized on a UV transilluminator (Vilber Lourmat, Torcy, France). Gels were photographed on instant black and white Polaroid film (number 667) using a hand-held Polaroid camera.

#### Statistical analysis of data

Data analysis was in accordance with the general linear model (GLM) of the system of analytical statistics (SAS). Means separation was with the least significant difference test (LSD) at 0.05 probability (Steel and Torrie, 1980).

## Results

#### Determination of the seed-borne nature of *V. dahliae* in olive

*Verticillium dahliae* was the causal agent of wilt as shown by its frequent isolation from wilted olive trees at different orchard-growing sites in Jordan. The fungus was identified by its cultural characteristics, growth pattern, colony morphology and by microscopic examination (Issac, 1967). When isolates were grown on the semi-selective medium or on PDA, the morphological structures typical for *V. dahliae* emerged: white cottony mycelium with verticillate branched conidiophores, ovoid conidia and black microsclerotia.

Seeds from *V. dahliae*-infected olive trees were generally normal in appearance, and seedlings that subsequently tested positive in the seed detection assays were indistinguishable from non-infected seedlings 8 weeks after planting. The percentages of infected seeds and seedlings are summarized in Table 1. The incidence of infection as determined by the nested PCR was significantly higher than that found with culture plating, from both symptomatic and asymptomatic infected trees of both olive cultivars, but *V. dahliae* infection was significantly

Table 1. Incidence of *Verticillium dahliae*-infected seeds and progeny seedlings from symptomatic and asymptomatic trees of olive cv. Shimlali and Nabali.

Cultivar	Infected seeds (%)				Infected progeny seedlings				LSD <sup>a</sup> value ( $P \leq 0.05$ )
	Symptomatic trees		Asymptomatic trees		Symptomatic trees		Asymptomatic trees		
	Isolation	Nested PCR	Isolation	Nested PCR	Isolation	Nested PCR	Isolation	Nested PCR	
Shimlali	15.0 a <sup>b</sup>	66.7 a	7.0 a	16.3 a	83.3 a	92.0 a	17.0 a	35.0 a	3.9
Nabali	11.3 b	63.3 a	7.3 a	13.3 a	73.0 b	91.3 a	8.0 b	23.0 b	2.8

<sup>a</sup> LSD, least significant difference.

<sup>b</sup> Means of 3 trees with 25 fruits/tree. Values followed by the same letter within columns are not significantly different according to the least significant difference test ( $P \leq 0.05$ ).

more frequent with symptomatic than with asymptomatic infected trees and it was in general more common with cv. Shimplali than with cv. Nabali (Table 1). Similar results were obtained with the progeny seedlings in both assays, and with both symptomatic and asymptomatic trees of both cultivars. The incidence of *V. dahliae* infection in the progeny seedlings was significantly higher than that in the seeds (Table 1).

#### Testing primer specificity for *V. dahliae* DNA detection

With the first amplification reaction, nested PCR contained primers NESF18S and NESR28S from the highly conserved DNA sequences of the 18S and 28S genes that flank the ITS regions. The second reaction was carried out under more stringent conditions (see below) that allowed specific amplification of *V. dahliae* DNA. Figure 1 shows the agarose electrophoresis gels of the products of each amplification reaction of nested PCR using DNA from mycelium extracts of different *V. dahliae* isolates. The first reaction comprised 35 cycles and produced the expected band size of around 480 bp for all isolates (Fig. 1A). The reaction prod-

ucts of the first amplification were used as template DNA for the second amplification reactions using primers FVD and RVD. It was necessary to decrease the number of cycles of the first nested reaction to optimize detection. In the second reaction, multiple bands were produced with a major band of the expected size of 330 bp (Fig. 1B). The additional bands may have been related to the high amount of template DNA. However these bands were significantly reduced in subsequent amplifications by increasing the annealing temperature of the two reactions and decreasing the number of cycles to 20 (Fig. 1B).

#### Testing nested PCR sensitivity for *V. dahliae*

Expected DNA fragments were amplified from all concentrations of the conidial suspension ( $2 \times 10^4$ ,  $2 \times 10^3$ ,  $2 \times 10^2$ , 20 and 2 conidia  $\mu\text{l}^{-1}$ ) using the two primer sets despite the direct use of conidia without DNA extraction (Fig. 3). A faint or no amplification signal of the expected size was produced after the first round (Fig. 3A). A thick, typical DNA band (330 bp) was produced in the second round of nested PCR even though the number of conidia per reaction in the first round decreased to 2 conidia  $\mu\text{l}^{-1}$ .

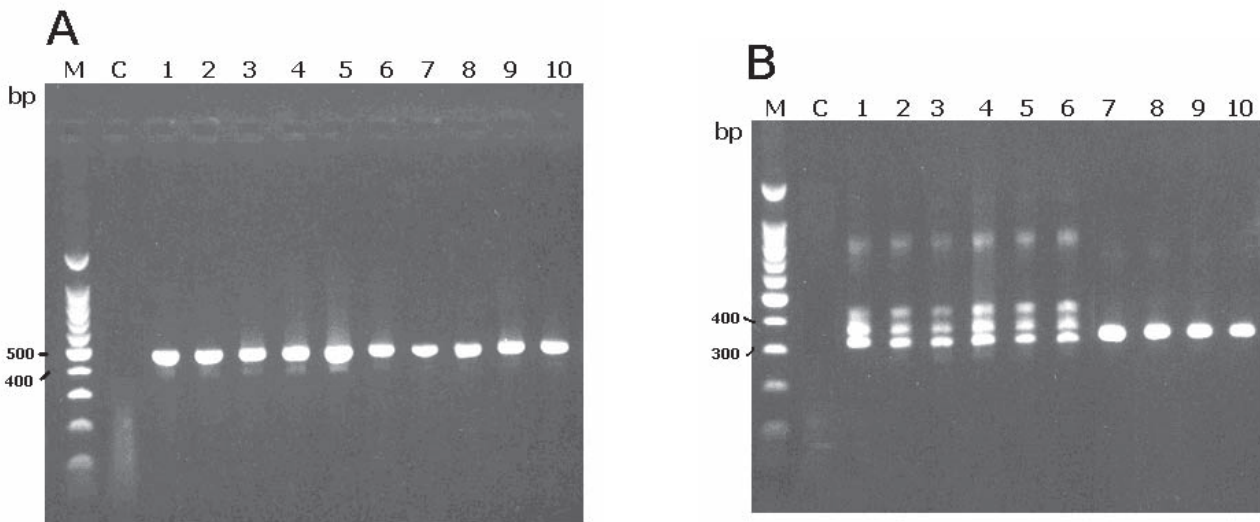


Fig. 1. Agarose gel electrophoresis of nested PCR products amplified by primers NESF18S and NESR28S in the first reaction of 40 cycles (A), and by primers FVD and RVD in the second reaction (B) from DNA extracts of the mycelium of *Verticillium dahliae* isolates (lane 1–6 using DNA products from the first reaction of 40 cycles; lane 7–10 using DNA products from the first reaction of 25 cycles); MU1, MU6, MU12, MU19, MU17, MU23, MU28, MU30, MU39, MU41, MU47, and MU70, respectively. Lane C, control (no added template). Lane M, 100 bp DNA marker.

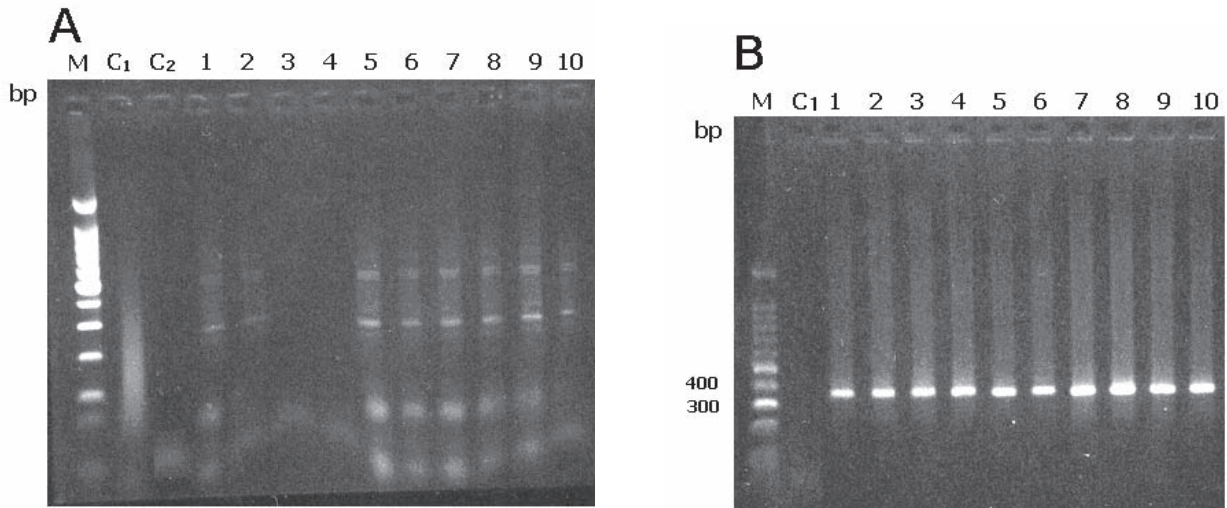


Fig. 2. Agarose gel electrophoresis of nested PCR products obtained with the universal primers in the first reaction of 40 cycles (A), and with primers FVDITS1 and RVDITS2 in the second reaction (B) from DNA extracts of several olive seeds from asymptomatic fruits (lane 1–10). Lane C<sub>1</sub>, control (no added template). Lane C<sub>2</sub>, control (olive DNA extract). Lane M, 100 bp DNA marker.

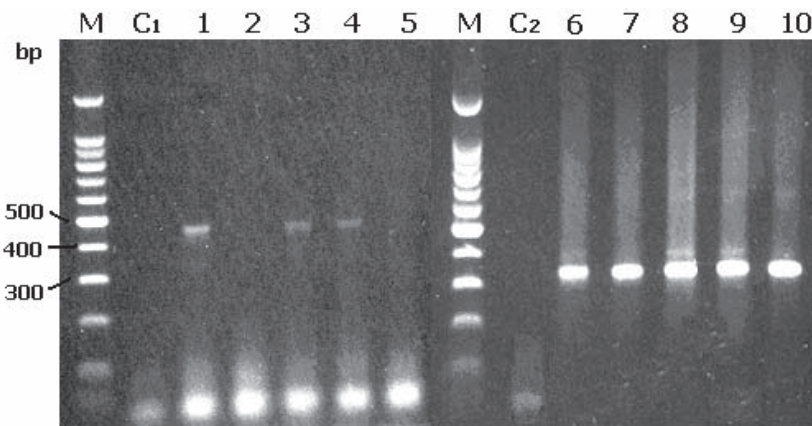


Fig. 3. Agarose gel electrophoresis of nested PCR products obtained with the NESF18S and NESR28S primers in the first reaction of 40 cycles (lane 1–5), and with primers FVD and RVD in the second reaction (lane 6–10) from conidial suspensions of *Verticillium dahliae* ( $2 \times 10^4$ ,  $2 \times 10^3$ ,  $2 \times 10^2$ , 20 and 2 conidia  $\mu\text{l}^{-1}$ ) used in the first round (lane 1–5) and second round (lane 6–10); lane C<sub>1</sub>, and C<sub>2</sub>, control, water (no added DNA template or conidia) in the first and second rounds; lane M, 100 bp DNA marker.

#### Nested PCR assay of olive seed and progeny seedlings

Total DNA was extracted and PCR performed on seeds and on progeny seedlings grown from seeds taken from symptomatic and asymptomatic trees (Table 1). No typical amplification signal of the expected size was produced with primers NESF18S and NESR28S after the first round of

the PCR (Fig. 2A). However, most of the samples gave a band of approximately 480 bp, as is expected when using this pair of universal primers. The PCR signals specific for *V. dahliae* were generated from DNA extracted from the seeds and progeny seedlings in the second round of the nested PCR with the primers FVD and RVD (Fig. 2B), confirming the presence of the pathogen. The spe-

cific signal for *V. dahliae* was obtained in all cases (Fig. 2). The olive seeds and progeny seedlings from which no DNA was amplified also did not yield any *V. dahliae* colonies on the semi-selective medium.

## Discussion

Several possible ways in which *Verticillium* wilt spreads to new olive growing areas have been suggested (Thanassouloupoulos *et al.*, 1984; Naser, 1996). Since *V. dahliae* occurs in the leaves of infected olive trees, leaves from those trees fallen on the ground can form microsclerotia in their petioles, increasing the amount of inoculum and contributing to disease spread (Tjamos and Botseas, 1987; Naser, 1996). Spinach and sugar beet seeds sown in soil infested with *V. dahliae* and grown to flowering produced infected seeds. *V. dahliae* could also be introduced into the soil by planting infected spinach seed, and the soil microflora may influence the transmission of *V. dahliae* by seed, and the subsequent development of wilt symptoms (Toit *et al.*, 2005).

*Verticillium dahliae* was recovered from seed, including seed from symptomless mother plants. Internal infection of seed with *V. dahliae*, particularly if the seed is from symptomless plants, may help the pathogen to spread to new areas because in Jordan growers usually collect olive seeds particularly those of the cultivar Shimlali, for planting them elsewhere as seedling rootstock.

To our knowledge this is the first report of seed transmission of *V. dahliae* in olive. Pathogen movement of *V. dahliae* through the vascular system of the fruit and into the seed has been reported in other vegetable crops (Vallad *et al.*, 2005; Toit *et al.*, 2005) and is also known for other vascular wilt pathogens (Gracia-Garza *et al.*, 1999).

Early, rapid, and reliable *in planta* detection of *V. dahliae* can help growers to avoid planting infected olive seed or seedlings and can thus facilitate the management of *Verticillium* wilt in olive. The diagnosis of *Verticillium* wilt in olive cannot be based on symptoms alone, however, but requires the isolation of *V. dahliae* from affected tissues. The isolation of *V. dahliae* from symptomatic olive branches is often erratic and sometimes unsuccessful (Naser, 1996).

In this study, specific primers were used in a

nested PCR procedure that detected *V. dahliae* in the seeds of symptomatic and asymptomatic nursery-propagated olive plants. Nested PCR analyses of these isolates showed that they were the same as the *V. dahliae* populations currently causing olive wilt in Jordan (Karajeh and Masoud, 2003). The nested PCR procedure was highly sensitive and did not rely on DNA extraction for DNA amplification by PCR, but used conidia directly at certain concentrations in the PCR reaction mix. This procedure in part resembled the procedure used to identify root-knot nematodes, in which single second-stage *Meloidogyne* juveniles were crushed in sterile water and their lysate was directly subjected to PCR also without DNA extraction (Harris *et al.*, 1990). Crushing of conidia was not necessary here because *V. dahliae* conidia are usually hyaline with a soft cell wall which allows the constituents to dissolve and to detect free DNA molecules under the stringent conditions of the PCR. The assay detected as few as 2 conidia per reaction mix, a sensitivity impossible with standard DNA extraction procedures, or by any other means. Nested PCR has also been used for the simultaneous detection of defoliating and non-defoliating *V. dahliae* pathotypes in olive plants (Mercado-Blanco *et al.*, 2003). The direct use of conidia in nested PCR may be useful in differentiating between defoliating and non-defoliating *V. dahliae* pathotype isolates without the need to extract genomic DNA, thus minimizing time, labor, cost and the risk of cross-contamination.

DNA samples were taken at different infection stages of the plant to challenge the improved detection procedure. In the nested PCR assays, the predicted PCR products obtained after the first reaction of nested PCR were not clearly visible, suggesting that pathogen DNA reached a maximum concentration in olive grown from infected seed.

The nested PCR-based assay was highly sensitive and detected *V. dahliae* in olive trees of different cultivars, varying in age, and growing under different conditions. This method detected a greater proportion of infections than did conventional pathogen isolation and, more importantly, it revealed the infecting pathogen, which isolations did not do. Furthermore, identification with culture plating requires 7 to 12 days, whereas molecular identification does take not more than

48 hours. With nested PCR, *V. dahliae* was detected in olive seed. The use of pathogen-free seed (i.e., seed tested to be free of the pathogen) for growing seedling root-stocks is an essential requirement for integrated disease management.

The isolation of the pathogen from the seed suggested that *V. dahliae* invaded the seed through the vascular system of the plant. However, because the pathogen was not recovered from any fruit part except the seed, the infection route may have been a different one. Histological studies should be undertaken to elucidate the precise location of *V. dahliae* (i.e., embryo, endosperm or cotyledons) and the route by which it penetrates the olive seed.

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